IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. No.	: 09/806,370	Confirmation No.: 8568
Applicant	: Holmes et al.	
Filed	: October 3, 2001	
TC/A.U.	: 1645	
Examiner	: V. Portner	
Customer No.	: 00270	
Title	: MUTANT CHOLERA HOLOTOXIN AS AN ADJUVANT	

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION

Sir:

I, Mary E. Bak, residing at 1415 Comly Court, Maple Glen, PA, 19002, a citizen of the United States of America, do declare and state:

1. I am one of the named attorneys of record in the above-identified patent application.

2. This Declaration is submitted in the above-identified application in response to the Examiner's rejection under 35 USC § 112, first paragraph in the Office Action dated November 15, 2005. This Declaration is submitted in compliance with 37 C.F.R. §1.57(f) to add to the present specification the amino acid sequence of SEQ ID NO: 1, previously entered as part of the July 12, 2004 amendments by asserting that this incorporation by reference was proper and does not introduce new matter into the specification.

EXPRESS MAIL NO: EU531733945US

3. Specifically, this Declaration is submitted to support the insertion by amendment of SEQ ID NO: 1 into the specification, which is an example of a mature wild-type cholera holotoxin subunit A sequence, as set forth in Domenighini et al., International Patent Publication No. WO 93/13202 (hereinafter Domenighini) cited in the specification at page 38, lines 10-27 and incorporated by reference. See, page 38 attached as **Exhibit A** herewith, where it is explicitly stated at lines 10-11: "International application WO93/13202 (36), which is incorporated by reference". Note that the Applicants referenced this publication not only to describe a series of mutations in the A subunit, but also to provide support for the nucleotide sequence encoding the A subunit of the cholera holotoxin, at page 38, lines 25-27 of the present specification, which recites "The nucleotide sequence encoding the A subunit of the cholera holotoxin is set forth in International application WO 93/13202.". This nucleotide sequence shown in Figs. 2a and 2b of Domenighini also displays the encoded mature amino acid sequence, which is illustrated in Figs. 1, 2a and 2b of Domenighini. Figs. 1, 2a and 2b of Domenighini are attached hereto as **Exhibit B**. That encoded amino acid sequence was inserted into the present specification as SEQ ID NO: 1 by way of the amendment filed on July 12, 2004. Applicants submit that the incorporation by reference of the nucleotide sequence of Figs. 2a and 2b of Domenighini is sufficient to implicitly incorporate the encoded amino acid sequence of SEQ ID NO: 1, because Figs. 2a and 2b of Domenighini also disclose the same amino acid sequence as that of Fig. 1 of Domenighini. Domenighini was cited as reference 2 in Applicants' Form PTO-1449, which was filed together with an Information Disclosure Statement on October 3, 2001, sent by Express Mail to Post Office Addressee service.

4. Declarant notes that a glutamic acid at amino acid position 29 of the mature A subunit of the wild-type cholera holotoxin appears in Figure 2 of Mekalanos et al., 1983, Nature, 306:551-557 (hereinafter Mekalanos), which is cited in the specification at page 2, line 4 (as Bibliography entry 1) in the context of the entire CT sequence with subunit B and 5' and 3' untranslated regions. Mekalanos was cited as reference 14 in Applicants' Form PTO-1449, which was filed together with the aforementioned Information Disclosure Statement on October 3, 2001. The mature

subunit A is indicated in Mekalanos by the number "1" appearing under the first mature amino acid "Asn" in the sequence. See, **Exhibit C** which is page 553 of Mekalanos, with the mature subunit A first amino acid highlighted and with the Glu at position 29 of the mature subunit A highlighted. SEQ ID NO: 1 is a mature subunit A sequence as set forth in both Domenighini and Mekalanos.

5. The sequence of SEQ ID NO: 1 identified in paragraph 3 above was added by way of the amendment filed on July 12, 2004. That amendment is now supported by this amended Declaration.

6. The sequence of SEQ ID NO: 1 identified in paragraph 3 is the previously added sequence of Domenighini. Therefore, in compliance with 37 C.F. R. §1.57(f): The material being inserted is the material previously incorporated by reference and the amendment contains no new matter.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: February 15, 2006

By: Mary E. Bak

Mary E. Bak

acid at position 29 of the A subunit of the cholera holotoxin, in combination with a selected antigen from a pathogenic bacterium, virus, fungus or parasite, is used to prepare an antigenic composition, wherein said holotoxin enhances the immune response in a vertebrate host to said antigen.

The antigenic compositions of this invention also comprise CT-CRM containing at least one additional mutation at a position other than at amino acid residue 29. International application WO 93/13202 (36), which is hereby incorporated by reference, describes a series of mutations in the A subunit which serve to reduce the toxicity of the cholera holotoxin. These mutations include making substitutions for the arginine at amino acid 7, the aspartic acid at position 9, the arginine at position 11, the histidine at position 44, the valine at position 53, the arginine at position 54, the serine at position 61, the serine at position 63, the histidine at position 70, the valine at position 97, the tyrosine at position 104, the proline at position 106, the histidine at position 107, the glutamic acid at position 110, the glutamic acid at position 112, the serine at position 114, the tryptophan at position 127, the arginine at position 146 and the arginine at position 192. The nucleotide sequence encoding the A subunit of the cholera holotoxin is set forth in International application WO 93/13202. International application WO 98/42375 (37) which is hereby incorporated by reference, describes making a substitution for the serine at amino acid 109 in the A subunit, which serves to reduce the toxicity of the cholera holotoxin. Therefore, using conventional techniques, mutations at one or more of these additional positions are generated.

Exhibit A to Declaration of Mary E. Bak
Dated: February 15, 2006

```
LT2      1  -.-.FF-----T----R-A---L---QQ-AYE---PI---     38
LT1      1  ------------------FRS-----.-------------     39
LT1_1A   1  -G-R-------------R-------HN-------------     40
CT       1  NDDKLYRADSRPPDEIKQSGGLMPRGQSEYFDRGTQMNIN     40

            --E-----V--NT--N------TVT--Q---I--N--GS-     78
            ----------------------------------Y-----     79
            --------------Y--------L-------A--S----Y     80
            LYDHARGTQTGFVRHDDGYVSTSISLRSAHLVGQTILSGH     80

            NE-----V-P---L-D--G---R---Y-S-N-FA------    118
            -LTIYI---...--------IS------------------    116
            ----------------------V-----Y-----------    120
            STYYIYVIATAPNMFNVNDVLGAYSPHPDEQEVSALGGIP    120

            L---I-----SF-A-EGGMQ---D--GDLF-G-TV--N--    158
            ----------------------------------------    156
            ----------N---I--R-----E------R--N----E-    160
            YSQIYGWYRVHFGVLDEQLHRNRGYRDRYYSNLDIAPAAD    160

            --Q-----SNFP----M--STF--EQ-VPNNKEFK-GV-I    198
            ----------------------------------------    196
            --R------D-Q------------Q---DSS-TITGD--N    200
            GYGLAGFPPEHRAWREEPWIHHAPPGCGNAPRSSMSNTCD    200

            SA-NV--KYD-MNFKKLL--RLALTFFM--D-F-GVHGE----    241
            --------------------------.-----.-----.----    236
            -E--N-STIY-R------------D-.--EV-.IY---.R---    240
            EKTQSLGVKFLDEYQSKVKRQIFSGY.QSDID.THNRI.KDEL    240
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Figure 1

Exhibit B to Declaration of Mary E. Bak
Dated: February 15, 2006

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LT  AATGGCGACAGATTATACCGTGCTGACTCTAGACCCCCAGATGAAATAAAACGTTTCCGG
    N  G  D  R  L  Y  R  A  D  S  R  P  P  D  E  I  K  R  F  R    20
    ------------------------------------------------------------
    N  D  D  K  L  Y  R  A  D  S  R  P  P  D  E  I  K  Q  S  G    20
CT  AATGATGATAAGTTATATCGGGCAGATTCTAGACCTCCTGATGAAATAAAGCAGTCAGGT

LT  AGTCTTATGCCCAGAGGT...AATGAGTACTTCGATAGAGGAACTCAAATGAATATTAAT
    S  L  M  P  R  G  Q  N  E  Y  F  D  R  G  T  Q  M  N  I  N    39
    ------------------------------------------------------------
    G  L  M  P  R  G  Q  S  E  Y  F  D  R  G  T  Q  M  N  I  N    40
CT  GGTCTTATGCCAAGAGGACAGAGTGAGTACTTTGACCGAGGTACTCAAATGAATATCAAC

LT  CTTTATGATCACGCGAGAGGAACACAAACCGGCTTTGTCAGATATGATGACGGATATGTT
    L  Y  D  H  A  R  G  T  Q  T  G  F  V  R  Y  D  D  G  Y  V    59
    ------------------------------------------------------------
    L  Y  D  H  A  R  G  T  Q  T  G  F  V  R  H  D  D  G  Y  V    60
CT  CTTTATGATCATGCAAGAGGAACTCAGACGGGATTTGTTAGGCACGATGATGGATATGTT

LT  TCCACTTCTCTTAGTTTGAGAAGTGCTCACTTAGCAGGACAGTATATATTATCAGGATAT
    S  T  S  L  S  L  R  S  A  H  L  A  G  Q  Y  I  L  S  G  Y    79
    ------------------------------------------------------------
    S  T  S  I  S  L  R  S  A  H  L  V  G  Q  T  I  L  S  G  H    80
CT  TCCACCTCAATTAGTTTGAGAAGTGCCCACTTAGTGGGTCAAACTATATTGTCTGGTCAT

LT  TCACTTACTATATATATCGTTATAGCA.........AATATGTTTAATGTTAATGATGTA
    S  L  T  I  Y  I  V  I  A           N  M  F  N  V  N  D  V    96
    ------------------------------------------------------------
    S  T  Y  Y  I  Y  V  I  A  T  A  P  N  M  F  N  V  N  D  V    100
CT  TCTACTTATTATATATATGTTATAGCCACTGCACCCAACATGTTTAACGTTAATGATGTA

LT  ATTAGCGTATACAGCCCTCACCCATATGAACAGGAGGTTTCTGCGTTAGGTGGAATACCA
    I  S  V  Y  S  P  H  P  Y  E  Q  E  V  S  A  L  G  G  I  P    116
    ------------------------------------------------------------
    L  G  A  Y  S  P  H  P  D  E  Q  E  V  S  A  L  G  G  I  P    120
CT  TTAGGGGCATACAGTCCTCATCCAGATGAACAAGAAGTTTCTGCTTTAGGTGGGATTCCA
```

Figure 2a

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LT   TATTCTCAGATATATGGATGGTATCGTGTTAATTTTGGTGTGATTGATGAACGATTACAT
     Y  S  Q  I  Y  G  W  Y  R  V  N  F  G  V  I  D  E  R  L  H     136
     ------------------------------------------------------------
     Y  S  Q  I  Y  G  W  Y  R  V  H  F  G  V  L  D  E  Q  L  H     140
CT   TACTCCCAAATATATGGATGGTATCGAGTTCATTTTGGGGTGCTTGATGAACAATTACAT

LT   CGTAACAGGGAATATAGAGACCGGTATTACAGAAATCTGAATATAGCTCCGGCAGAGGAT
     R  N  R  E  Y  R  D  R  Y  Y  R  N  L  N  I  A  P  A  E  D     156
     ------------------------------------------------------------
     R  N  R  G  Y  R  D  R  Y  Y  S  N  L  D  I  A  P  A  A  D     160
CT   CGTAATAGGGGCTACAGAGATAGATATTACAGTAACTTAGATATTGCTCCAGCAGCAGAT

LT   GGTTACAGATTAGCAGGTTTCCCACCGGATCACCAAGCTTGGAGAGAAGAACCCTGGATT
     G  Y  R  L  A  G  F  P  P  D  H  Q  A  W  R  E  E  P  W  I     176
     ------------------------------------------------------------
     G  Y  G  L  A  G  F  P  P  E  H  R  A  W  R  E  E  P  W  I     180
CT   GGTTATGGATTGGCAGGTTTCCCTCCGGAGCATAGAGCTTGGAGGGAAGAGCCGTGGATT

LT   CATCATGCACCACAAGGTTGTGGAGATTCATCAAGAACAATCACAGGTGATACTTGTAAT
     H  H  A  P  Q  G  C  G  D  S  S  R  T  I  T  G  D  T  C  N     196
     ------------------------------------------------------------
     H  H  A  P  P  G  C  G  N  A  P  R  S  S  I  S  N  T  C  D     200
CT   CATCATGCACCGCCGGGTTGTGGGAATGCTCCAAGATCATCGATCAGTAATACTTGCGAT

LT   GAGGAGACCCAGAATCTGAGCACAATATATCTCAGGGAATATCAATCAAAAGTTAAGAGG
     E  E  T  Q  N  L  S  T  I  Y  L  R  E  Y  Q  S  K  V  K  R     216
     ------------------------------------------------------------
     E  K  T  Q  S  L  G  V  K  F  L  D  E  Y  Q  S  K  V  K  R     220
CT   GAAAAAACCCAAAGTCTAGGTGTAAAATTCCTTGACGAATACCAATCTAAAGTTAAAAGA

LT   CAGATATTTTCAGACTATCAGTCAGAGGTTGACATATATAACAGAATTCGGGATGAATTATGA
     Q  I  F  S  D  Y  Q  S  E  V  D  I  Y  N  R  I  R  D  E  L  *
     ---------------------------------------------------------------
     Q  I  F  S  G  Y  Q  S  D  I  D  T  H  N  R  I  K  D  E  L  *
CT   CAAATATTTTCAGGCTATCAATCTGATATTGATACACATAATAGAATTAAGGATGAATTATGA
```

Figure 2b

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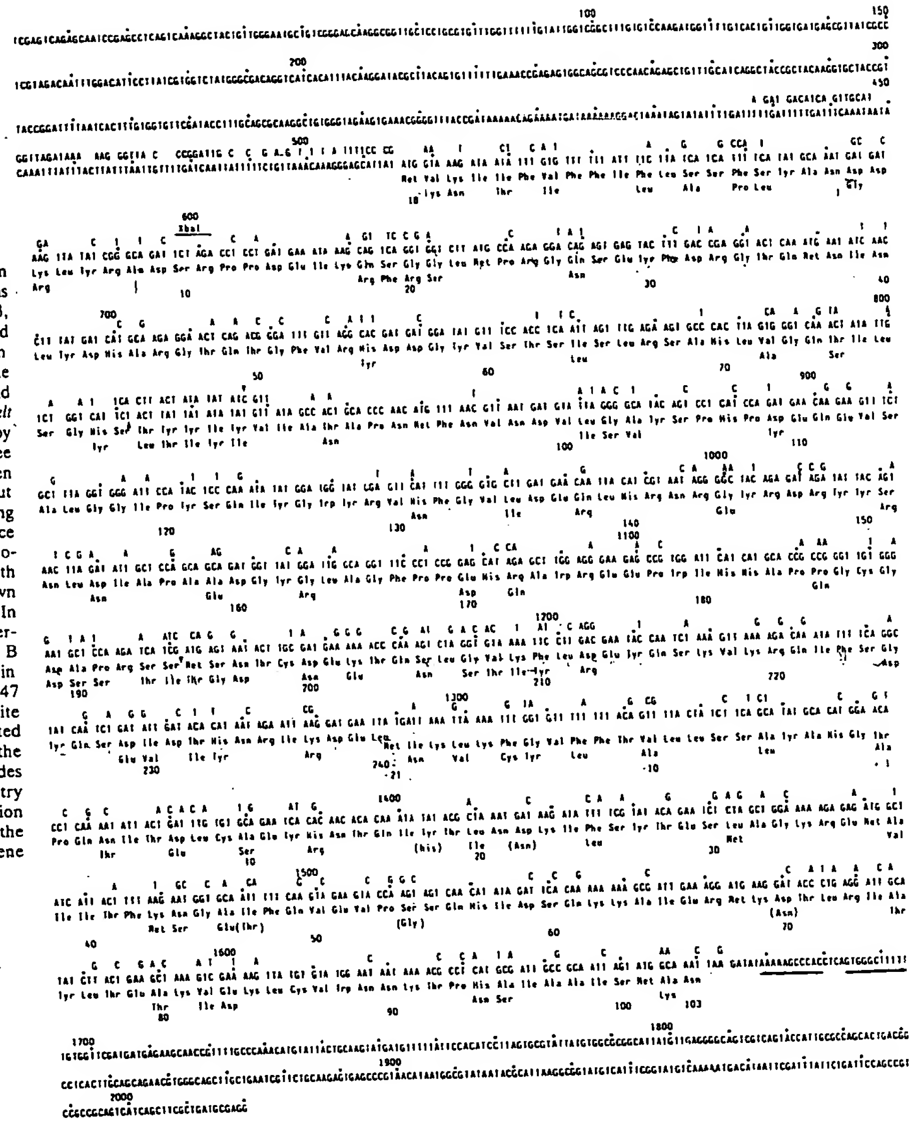

Fig. 2 DNA sequence of the *V. cholerae* toxin operon from strain 2125. The antisense strand is shown from 5′ to 3′. From nucleotide 427 to 1,663, the sequence is compared with the published sequence of LT genes[20,21]; *elt* nucleotides are shown above the sequence only where they differ from the *ctx* sequence except between nucleotides 810 and 830, where deletion of a T (arrowed) in the *elt* sequence creates a frameshift which is corrected by insertion of a C (arrowed) 16 bp downstream (see text). Analogous events have previously been seen after pseudoreversion of frameshift mutations[45] but to our knowledge, this is the first naturally occurring case described. The deduced amino acid sequence of *ctxA* (nucleotide 516 to 1,289) and *ctxB* (nucleotide 1,289 to 1,660) is shown and compared with that of LT. Amino acids that differ in LT are shown below the cholera toxin amino acid sequence. In addition, for the mature B subunit sequence, differences from the published amino acid sequence of B subunit purified from strain 569B[26,27] are shown in brackets. Two of these differences (amino acids 47 and 54) are also found in LT. The cleavage site between the A1 and A2 polypeptides is indicated by an arrow (amino acids 194–195). Note also the overlap of *ctxA* and *ctxB* cistrons (nucleotides 1,289–1,292). Sequence exhibiting dyad symmetry and potentially involved in transcription termination is indicated with divergent arrows. Features of the sequence immediately upstream of the *ctxA* gene are detailed in Fig. 3.

sequence (nucleotides 1,277–1,282, Fig. 2) of the *ctxA* cistron. The first two nucleotides of the *ctxA* translation termination signal TGA are the last two nucleotides of the *ctxB* translation initiation triplet ATG. This particular overlapping arrangement is also found several times in phage λ operons[30] and may be involved in translational coupling[31] of the *ctxA* and *ctxB* genes. However, evidence presented below suggests that this is not the case with the *ctx* operon. Where documented, translational coupling is observed between cistrons whose gene products interact in a one to one stoichiometry[31], and in contrast, the cholera toxin molecule is composed of one A subunit and five B subunits. Moreover, *E. coli* produces stoichiometrically 7 times more cholera toxin B subunit than A subunit (data not shown). Fusion of the *ctxB* gene to various *E. coli* promoters allows high expression of *ctxB* in the absence of *ctxA* translational initiation signals. These data suggest that translation of *ctxB* relies primarily on independent initiations promoted by its own ribosome binding site.

Another experiment supports this conclusion. Our DNA sequencing analysis identified two *Nde*I sites at positions 561 and 1,337 within the *ctxA* and *ctxB* genes, respectively. The positions of these sites relative to the reading frames of *ctxA* and *ctxB* allowed us to construct a *ctxA* deletion which codes for an in-frame fusion of amino acid 17 of the A subunit signal sequence to amino acid 19 of the B signal and thus maintains the normal processing site of the B signal sequence (residue 21). This genetic fusion makes B subunit expression dependent on the efficiency of the A cistron translation initiation sequences, provided the hybrid signal sequence is processed at normal efficiency. *Nde*I digestion of plasmid pGP3 followed by ligation produced such a fusion between these two sites and gave plasmid pJM3.1. Plasmid pJM3.1 produced 0.056 $\mu g\ ml^{-1}$ of B subunit in *E. coli* MS371 while pGP3 produced 0.50 $\mu g\ ml^{-1}$. These data suggest that the *ctxB* ribosome binding site is about ninefold more efficient than the *ctxA* site.

Toxin promoter regions

We determined approximately 200 base pairs of sequence upstream of the *Xba*I sites for each of the other five additional cloned copies of the *ctxA* gene, cloned on plasmids pGP3, pGP4, pGP5, pGP6 and JM17. Comparison of these sequences with the corresponding region of the *ctxA* gene derived from strain 2,125 indicated a perfect conservation of sequence between these copies from nucleotides 413 to 590 with one notable exception. The sequence TTTTGAT comprising nucleotides 419–425, 426–432 and 433–439 of the 2,125 sequence was found tandemly repeated 3–8 times preceding different *ctxA* gene copies (Fig. 3). Figure 3 shows part of a sequencing gel autoradiograph that spans DNA carrying eight of these tandem repeats in the region adjacent to the *ctxA* gene of pJM17.

To determine the position of the toxin operon promoter with respect to these repeated sequences, we used nuclease *Bal*31

Exhibit C to Declaration of Mary E. Bak
Dated: February 15, 2006